PCR and Genotyping for HPV in Cervical Cancer Patients

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ABSTRACT

Aims: To devise nested multiplex polymerase chain reaction (NMPCR) protocol for detection of mucosal human papilloma viruses (HPVs) and typing of HPV-16 and -18 in formalin-fixed, paraffin-embedded (FFPE) tissues of carcinoma cervix (CaCx). **Settings and Design:** Cross-sectional observational study. **Materials and Methods:** NMPCR was done for simultaneous detection of HPV, targeting 134 bp L1 capsid gene employing GP+/mGP+ primers and typing of genotypes-16 and -18, targeting E6/E7 gene from 34 FFPE tissue blocks of CaCx and cervical intraepithelial neoplasia (CIN). Detection of 142 bp consensus sequence of L1 capsid gene was performed by nested PCR employing MY/GP+ primers. Sequencing of selected PCR amplicons of the later protocol obtained from control cell line DNA and 5 select samples were done for validation of the NMPCR protocol. **Statistical Analysis Used:** Calculation of percentage from the Microsoft Excel Software. **Results:** Of 26 FFPE samples of CaCx, 17 (65.3%) samples were found positive for HPV by NMPCR. Amplicons of 142 bp L1 capsid gene employing MY/GP+ primers were observed in 11 (42.3%) samples of CaCx. Nearly 25% samples of CIN were positive for HPV. On sequence analysis, it was observed that the sample typed as HPV-16 by NMPCR was found to be the same on sequencing of amplicons obtained after MY/GP+ nested PCR. **Conclusions:** This study indicates the usefulness of our NMPCR protocol for detection of mucosal HPVs and typing of HPV-16 and -18 from FFPE tissue samples of CaCx. The NMPCR protocol may be used to detect HPV and type common genotypes-16 and -18 in fresh tissue of cervical biopsy or scrape samples for screening of CaCx.

Key words: Cervical intraepithelial neoplasia, molecular diagnosis, Pap smear, polymerase chain reaction, squamous cell carcinoma

INTRODUCTION

Globally, carcinoma cervix (CaCx) is the fourth most common cancer in women and seventh overall, with an estimated 528,000 new cases in 2012.[1] In India, 122,844 women are diagnosed with CaCx every year with about 55% mortality rate.[2] India shares about 25.4% morbidity and 26.5% mortality of the global load of CaCx, respectively.[3] Infection with human papillomavirus (HPV) is necessary but not a sufficient event in cervical carcinogenesis.[4] There are more than 100 types of HPV, of which forty types are known to infect the genital tract and spread through sexual contact.[5] Among these, persistent infection/integration of any of the 15 genotypes, namely, HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -68, -73, and -82 are implicated in causation of CaCx and are designated as high-risk (HR) HPVs.[6] HPV-16 and-18 together are responsible for about 70% cases of CaCx occurring in every region of the world.[7] It has been observed that HPV infections are more likely to persist in HIV-positive women and in this subset of women, HPV-16 remained underrepresented and showed a higher proportion of other HR HPV types

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compared with the general female population with high grade squamous intraepithelial lesions (HSILs).[8]

Cervical HPV infection is contracted by mucosal contact during sexual intercourse and it is commonly present in young women after the onset of sexual activity. The majority of cervical HPV infections get spontaneously cleared within a few years.[9] However, the longer the duration of cervical HPV infection (persistence), the lesser are the chances that a patient can clear his/her infection. It has been observed that <10% of new cervical HPV infections become persistent, lead to dysplastic changes of the cervical epithelium, and progress to precancerous lesions, typically over a period of 5-10 years.[10] In a minority of women with precancerous lesions, CaCx arises over many years with a peak or plateau in risk at about 35-55 years of age. Thus, HPV-associated cervical carcinogenesis progresses from HPV infection to HPV persistence to the development of high-grade cervical intraepithelial neoplasia (CIN) and ultimately develops into CaCx; and it appears that on an average, this process may take up to 15 years.[11]

Cervical Pap smear test is the routinely used screening method for identification of HSILs (CIN-2 and 3) and CaCx; however, the diagnostic confirmation is obtained on histopathological examination of cervical biopsy samples. Squamous cell carcinoma (SCC) and adenocarcinoma are the two most common histological subtypes of CaCx and both are related to cervical HPV infection. In a hospital-based study from a tertiary care center of North India, the most common histological type of cervical cancer was SCC (92.5%) followed by adenocarcinoma (5.9%).[12] However, in comparison to cervical SCC, a rising trend towards cervical adenocarcinoma was noted in developed countries.[13] Nevertheless, cervical SCC and cervical adenocarcinoma show identifiable HPV sequences in more than 99% cases.[14,15]

Unlike other DNA viruses, conventional cell cultures cannot detect HPV. Based on the knowledge of genotype-specific changes in L1 and E6 region of the viral genome, different investigators have designed in-house polymerase chain reaction (PCR)/hybridization-based HPV detection and typing methods.[16,17] There are FDA-approved HPV kits, namely, Hybrid Capture II, Cervista™ HPV HR, Cervista™ HPV-16/-18 and Cobas® HPV Test for detection of HR HPV in DNA isolated from cervical samples. The recently introduced Kit Aptima HPV assay detects mRNA expressed by HR HPVs, thus making this test more specific than others.[18]

Recently, in April 2014, Cobas 4800 HPV test (Roche), which can detect HR HPVs as a pool and indicate HPV-16 and-18, if present, has been approved by the FDA for primary cervical cancer screening in women aged 25 years and older. The decision of FDA was based on findings of ATHENA study, in which Cobas 4800 HPV test was evaluated among 47,208 women of the United States aged 21 years and undergoing routine cervical cancer screening. Results from trial revealed that 1 in 10 women, aged 30 years and older, who tested positive for 16 and/or 18, actually had cervical precancer even though they showed normal results with the cervical Pap test.[19]

In the cluster-randomized trial, conducted by Sankaranarayanan *et al*., similar efficacy of HC2 assay meant for detection for HR-HPV panel and cytology in detection of preinvasive lesions was initially observed, however, later they concluded that in low-resource settings, even a single round of HPV testing may lead to significant reduction in number of advanced cervical cancer and deaths from it.[20] We have also witnessed similar results in our previous work.[21]

In this study, we devised nested multiplex PCR (NMPCR) to detect mucosal HPVs as a pool and simultaneously tried to type the two most common HPV genotypes-16 and-18, in formalin-fixed paraffin-embedded (FFPE) tissue samples of CaCx and CIN.

MATERIALS AND METHODS

A total of 34 FFPE tissue blocks, of histopathologically diagnosed samples of CaCx ($n = 26$) and CIN ($n = 8$) were retrieved from the archive of Department of Pathology for isolation of DNA- and PCR-based detection of HPV. These FFPE samples included cervical SCC ($n = 24$), cervical adenocarcinoma ($n = 2$), CIN grade-1 ($n = 4$), CIN grade-2 ($n = 1$), and CIN grade-3 ($n = 3$).

DNA isolation

DNA isolation from FFPE tissue was done using commercial kit QIAamp DNA FFPE Tissue kit (QIAGEN; Cat no: 56404). Briefly, 5 μm thick 10 sections of paraffin blocks were taken in an eppendorf tube, deparaffinized in xylene, and subsequently treated with 100% ethanol to remove xylene. The tissue pellet so received was dried up at room temperature, treated with 40 μL proteinase K, incubated at 56°C for 1 h and subsequently incubated at 37°C for overnight until the tissue was completely lysed. Next morning, the lysed tissue was incubated at 90°C

for 1 h. Thereafter, DNA was extracted employing mini elute column and buffers supplied with the kit. DNA was finally eluted in 40 μL of ATE buffer supplied with the kit. Quantity and quality of extracted DNA was checked by spectrophotometer (Nanodrop®) as well as by running on 0.8% agarose gel. After validating the DNA extraction results, DNA was stored at −20°C until PCR was performed.

Polymerase chain reaction-based detection of human papilloma virus

The HPV detection was attempted using two different protocols of nested PCR.

Simultaneous detection of human papilloma virus and typing of human papilloma virus-16 and -18 by nested multiplex polymerase chain reaction

The first PCR protocol, which is an NMPCR, was attempted for simultaneous detection of HPV, targeting 134 bp L1 capsid gene employing GP+/mGP+ primers and typing of genotypes-16 and 18, targeting E6/E7 gene employing primers described in Table 1.[16,22,23] The mGP + primers were designed by trimming bases from 5' ends of GP + primers for this study.

Master mix for first round PCR

Master mix (25 μl) for the first cycle of PCR was prepared by using 10X buffer (Merck, GeNei, Bengaluru, India), 10 mM dNTP mix (Merck, GeNei, Bengaluru, India), Taq polymerase (Merck, GeNei, Bengaluru, India), 10 pmol of consensus forward primer and reverse primers of both L1 and E6/E7 gene, i.e., GP5+, GP6+, GP-E6-3F, GP-E7-5B, GP-E7-6B (Eurofins, Bengaluru, India), 10 μl DNA template and deionized water (qs).

Table 1: Primers for amplification of human papilloma virus gene sequence

Serial number	Code	Oligo sequence	Expected size of amplicons
1	MY11	5'-GCM CAG GGW CAT AAY AAT GG-3'	452 bp
2	MY09	5'-CGT CCM AAR GGA WAC TGA TC-3'	
3	GP5+	5'-TTT GTT ACT GTG GTA GAT ACT AC-3'	142 bp
4	GP6+	5'-GAA AAA TAA ACT GTA AAT CAT ATT C-3'	
5	mGP5+	5'-GTT ACT GTG GTA GAT ACT AC-3'	134 bp
6	mGP6+	5'-A TAA ACT GTA AAT CAT ATT C-3'	
7	GP-E6-3F	5'-GGG WGK KAC TGA AAT CGG T -3'	630 bp
8	GP-E7-5B	5'-CTG AGC TGT CAR NTA ATT GCT CA-3'	
9	GP-E7-6B	5'-TCC TCT GAG TYG YCT AAT TGC TC-3'	
10	16 F	5'-CAC AGT TAT GCA CAG AGC TGC-3'	457 bp
11	16 R	5'-CAT ATA TTC ATG CAA TGT AGG TGT A-3'	
12	18 F	5'-CAC TTC ACT GCA AGA CAT AGA-3'	322 bp
13	18 R	5'-GTT GTG AAA TCG TCGTTT TTC A-3'	

Source: Primers 1-2: Bauer and Manos, 1993,[22] Primers 3-4: de Roda Husman *et al*., 1995,[23] Primers 5-6: Designed for the present study, Primers 7-13: Sotlar *et al*., 2004[16]

The master mix for the first cycle of PCR was subjected to forty cycles of amplification in the Thermal Cycler (Bio-Rad, Hercules, CA, USA); 1 min denaturation at 94°C, 1 min annealing at 42°C, and 1 min elongation at 72°C, with a final elongation step extended to 7 min.

Master mix for the second round polymerase chain reaction

Master mix (25 μl) for the second round of PCR contained the same constituents as described for the first cycle except the primers, mGP5+, mGP6+, 16F, 16R, 18F, and 18R (Eurofins, Bengaluru, India) were used, and only 1 μl product of first cycle was used as template.

The reaction mixture was again subjected to forty cycles of amplification in the Thermal Cycler (Bio-Rad, USA) at annealing temperature of 53°C.

Detection of 142 bp L1 capsid gene by nested polymerase chain reaction employing MY/GP+ primers

The second PCR protocol is nested PCR for detection of 142 bp L1 capsid gene by nested PCR employing MY/GP+ primers.

Primers

The MY/GP+ primers used for amplification of consensus sequence of L1 capsid gene by nested PCR is given in Table 1.

Master mix for the first round polymerase chain reaction

Master mix (25 μl) for the first round of PCR was prepared using 10X buffer (Merck, GeNei, Bengaluru, India), 10 mM dNTP mix (Merck, GeNei, Bengaluru, India), Taq polymerase (Merck, GeNei, Bengaluru, India), 10 pmol of consensus forward primer and reverse primers, i.e., MY11 and MY09 (Eurofins, Bengaluru, India), 10 μl DNA template and deionized water (qs).

The master mix for the first cycle of PCR was subjected to 45 cycles of amplification in the Thermal Cycler (Bio-Rad, USA) at annealing temperature of 47°C.

Master mix for the second round polymerase chain reaction

Master mix (25 μl) for the second round of PCR contained the same constituents as described for the first cycle except the primers, GP5+ and GP6+ (Eurofins, Bengaluru, India) was used and only 1 μl product of first cycle was used as template.

The reaction mixture was again subjected to forty cycles of amplification at annealing temperature of 53°C.

Positive control

DNA isolated from SiHa and HeLa 229 cell lines were used as positive control for HPV-16 and -18, respectively, as well as for consensus sequence PCR.

Amplicons obtained from both PCR protocols were subjected to 1.6% agarose gel electrophoresis along with positive control and 100 bp DNA ladder.

Sequencing of amplicons

The amplicons obtained from the selected samples after nested PCR targeting 142 bp L1 capsid gene using MY/GP+ primers and control SiHa and HeLa cell line DNA were submitted to Merck Specialities, Bengaluru, India, for sequencing, to validate the NMPCR protocol for simultaneous detection of HPV as well as typing of HPV-16/-18.

In silico analysis of LI sequences of human papilloma viruses

To analyze the ability of GP + primers to anneal with four HR HPVs (HPV-59, -68, -73, and -82) which were not analyzed by de Roda Husman *et al*.,[23] we used bioinformatics analyses using online multiple sequence alignment software ClustalW2(EMBL-EBI, Hinxton,Cambridgeshire,UK) to examine sequence similarity of L1 sequences of HR HPVs, namely, 59 (accession no. EU918767.1), 68 (accession no. EU918769.1), 73 (accession no. X94165.1), 82 (accession no. JN644141.1), along with 6 (accession no. FR751338.1), 11 (accession no. JN644141.1), 16 (accession no. K02718.1), and 18 (accession no. X05015.1).

Ethical issues

For this study, approval was obtained from the Institutional Ethics Committee.

RESULTS

In this study, PCR detection of HPV was performed on DNA that was isolated from FFPE tissues of diagnosed cases of CaCx and CIN. The sensitivity of nested PCR protocol was assessed by subjecting 10-fold serial dilutions of SiHa cell line DNA for detection of HPV-16 specific amplicons. After DNA isolation, the yield of DNA isolated from SiHa cell lines was 28 ng/μl and it was observed that nested PCR was able to detect HPV-16 specific sequence from the 10 μl-diluted DNA. The detection of HPV-16 was observed even after diluting it 10^3 times to the order of picogram (10^{-12} g).

Squamous cell carcinoma and human papilloma virus

On subjecting 24 samples of SCC of the uterine cervix for detection of HPV using NMPCR protocol for detection (GP5+/6+ and mGP5+/mGP6+) as well as typing of HPV-16 and -18 (E6 consensus and specific primers), it was observed that 17 samples were positive for HPV-specific amplicons [Figure 1] [Table 2]. On nested PCR detection of L1 capsid gene targeting 142 bp sequences, 11 out of 24 samples of SCC of uterine cervix yielded HPV-specific amplicons. These 11 samples were inclusive of three HPV-16 positive samples and typed by NMPCR [Figure 2].

Adenocarcinoma and human papilloma virus

None of the two FFPE samples of adenocarcinoma of uterine cervix yielded either HPV-16/-18 or L1 capsid-specific (134 bp) amplicons on NMPCR. Furthermore, these two samples did not produce L1 capsid product (142 bp) after nested PCR using MY09/MY11 and GP5+/GP6+ primers [Table 2].

Cervical intraepithelial neoplasia and human papilloma virus

Out of eight samples of CIN, only two samples (one each from CIN-1 and CIN-2) were positive for HPV and both of these were HPV-16 associated lesions.

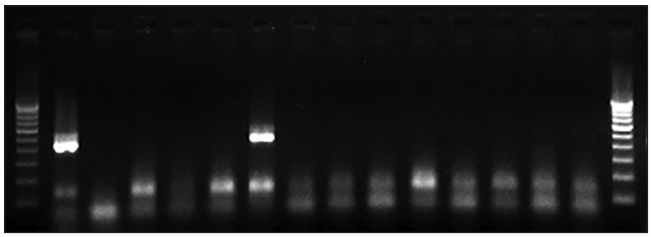

Figure 1: Gel picture of amplicons obtained after nested multiplex polymerase chain reaction for the detection of human papilloma virus. Lane 1 and 16: 100bp DNA ladder, Lane 2: Positive control (SiHa cell line) 457 bp E6 and 134 bp L1 amplicons, Lane 3: Negative control (Milli-Q water), Lane 4, 6, 7, 9-15: Carcinoma cervix positive for human papilloma virus (134 bp), Lane 7: Carcinoma cervix positive for human papilloma virus-16 (457 bp)

Table 2: Polymerase chain reaction detection of human papilloma virus in carcinoma cervix and preinvasive lesions (*n* = 34)

Lesion	Number of samples	NMPCR* E6/7 and GP+/mGP+		Nested PCR MY/GP+
		E6/E7 gene (457 bp HPV-16) *n* (%)	L1 capsid gene (134 bp) *n* (%)	L1 capsid gene (142 bp) *n* (%)
SCC†	24	3 (12.5)	17 (70.8)	11 (45.8)
ADC‡	2	0	0	0
CIN1§	4	1 (25.0)	1 (25.0)	1 (25.0)
CIN2‖	1	0	0	0
CIN3*	3	1 (33.3)	2 (66.6)	1 (33.3)

*NMPCR: Nested multiplex polymerase chain reaction, †SCC: Squamous cell carcinoma, ‡ADC: Adenocarcinoma, §CIN1: Cervical intraepithelial neoplasia grade-1, ‖CIN2: Cervical intraepithelial neoplasia grade-2, *CIN3: Cervical intraepithelial neoplasia grade-3, HPV: Human papilloma virus, PCR: Polymerase chain reaction

Both these CIN samples showed positive results on NMPCR detection of HPV-16/-18 and HPV-specific amplicons of L1 capsid gene (134 bp). These were also positive for nested PCR targeting 142 bp sequence of L1 capsid gene. The rest of the six CIN tissue samples have displayed negative results with both NMPCR and nested PCR [Table 2].

Sequence analysis of L1 capsid gene sequences

Sequence analyses revealed that 145 bp of HeLa and 142 bp of SiHa cell line L1 capsid gene PCR products showed 100% sequence similarity with sequences of HPV-18 (Sequence ID:Gb|EF140825.1|) and HPV-16 (Gb|KJ549650.1|), respectively, already present in NCBI database. Further, the status of HPV-16 genotype of a single sample of SCC of the uterine cervix, which yielded HPV-16 specific 457 bp amplicons and 134 bp L1 capsid amplicons by NMPCR, was also confirmed by sequencing of 142 bp MY/GP+ nested PCR product. Interestingly, MY/GP+ product of four randomly selected samples of CaCx which did not yield HPV-16/-18 specific product on NMPCR also turned out positive for HPV-16 on sequencing [Table 3].

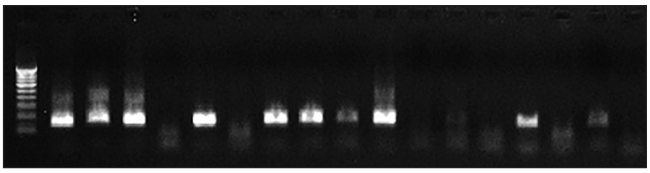

Figure 2: Gel picture of MY/GP nested polymerase chain reaction products of human papilloma virus positive carcinoma cervix. Lane 1: 100 bp DNA ladder, Lane 2, 3: Positive control (SiHa cell line and HeLa cell line DNA), Lane 16: Negative control (Milli-Q water), Lane 4, 6, 8, 9, 10, 11, 15: Carcinoma cervix positive for human papilloma virus (142 bp),

In silico analysis of L1 sequences of human papilloma viruses

On analyzing the sequence similarity of L1 sequences of HR HPVs, namely, 59, 68, 73, 82 along with 6, 11, 16, and 18 and sequences of amplicons of obtained with GP + primers on DNA isolated from HPV-16 transfected cell line SiHa (HPV-16a) and HPV-18 transfected cell line HeLa (HPV-18a), using ClustalW, it has been observed that terminal 2 bases of 3' end of both forward and reverse primers, namely, GP5+ and GP6+, were common in all the genotypes [Figure 3].

DISCUSSION

HPV was considered as the most important risk factor in the pathogenesis of CaCx after the demonstration of integrated HPV-16 and -18 genome in the biopsy specimens and cell lines derived from HIV-positive CaCx in the early 1980s.[24,25] Until recently, HPV-16 and -18 continues to be the two most common types of HPV isolated from DNA samples of CaCx.[7] As per recent estimates, more than

Table 3: Sequencing of MY/GP+ nested polymerase chain reaction product of cell lines and select carcinoma cervix samples

Sample number	Sample	Lab code	Genotype 16/18 detected by NMPCR*	HPV type and size of amplicons obtained on sequencing	Sequence similarity
1	HeLa 229 cell line	HeLa HPV 18 control	HPV 18	HPV 18 145 bp	100% with HPV 18 Sequence ID: gb|EF140825.1|
2	SiHa cell line	SiHa HPV 16 control	HPV 16	HPV 16 142 bp	100% with HPV 16 Sequence ID: gb|KJ549650.1|
3	SCC†	Cx 2	Not detected	HPV 16 143bp insertion of cytosine at 98 (accession number KU961844)	99.3% with HPV 16 Sequence ID: gb|KJ549650.1|
4	SCC†	Cx 8	HPV 16	HPV 16 142 bp C replaces A at 42 (accession number KU961845)	99.29% with HPV 16 Sequence ID: gb|KJ549650.1|
5	SCC†	Cx 10	Not detected	HPV 16 142 bp C replaces A at 42 (accession number KU961846)	99.29% with HPV 16 Sequence ID: gb|KJ549650.1|
6	SCC†	Cx 15	Not detected	HPV 16 142 bp (accession number KU961847)	100% with HPV 16 Sequence ID: gb|KJ549650.1|
7	SCC†	Cx 25	Not detected	HPV 16 142 bp C replaces A at 24 (accession number KU961848)	99.29% with HPV 16 Sequence ID: gb|KJ549650.1|

All these samples were positive for 134 bp by GP+/mGP + nested PCR. *NMPCR: Nested multiplex polymerase chain reaction, †SCC: Squamous cell carcinoma. PCR: Polymerase chain reaction

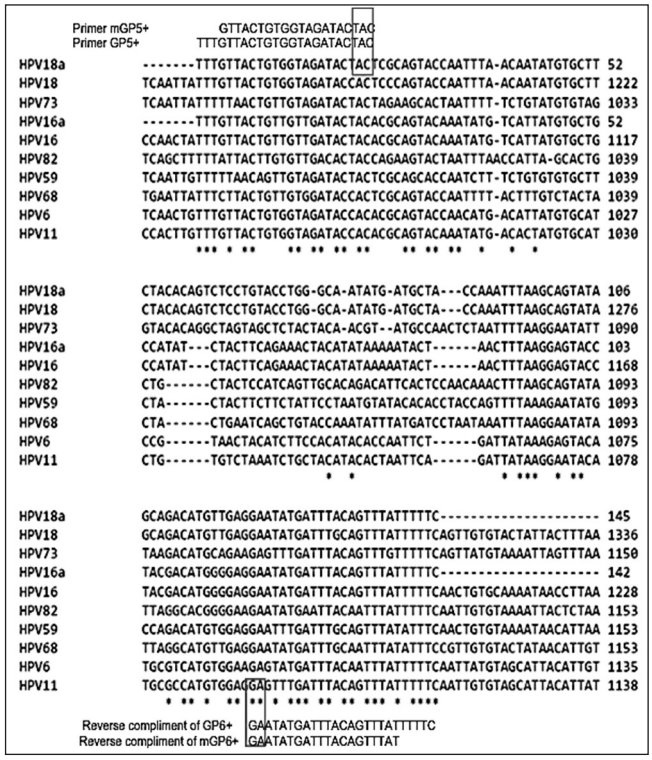

Figure 3: Sequence analyses for L1 gene sequence for GP+ primers compatibility

0.8 million women in India are HIV positive.[26] Further, the importance of detection of cervical infection with HR HPVs in the screening of CaCx has been emphasized among this subset of women.[27] In a study conducted in western part of India, it was observed that the HPV genotype distribution in CIN among HIV-infected women, the HR HPV genotypes in declining order of prevalence were HPV-16, -56, -18, -39, -35, -51, -31, -59, -33, -58, -68, -45, and -52.[28] In a similar study from North India, among HIV HPV co-infected females, HPV-16 was the most common type, detected only in 42% of HPV-positive women, followed by HPV-45 (15%), HPV-18/-52/-31/-58 (11.5% each), and HPV-33 (7.6%). The corresponding figures in the control group were as follows: HPV-16 (66.6%), HPV-45/-18/-31 (16.6% each), and HPV-33/-58/-68 (8.3% each).[29] These observations indicate that almost all HR HPVs are circulating in Indian population. Further, it has been noted that HIV co-infection in HPV infected females leads to dysregulation of the cellular and humoral arms of the local and systemic immune systems ensuring HPV-associated aggressive cancer progression through the microsatellite instability pathway instead of loss of heterozygosity observed among HIV seronegative females.[30] Thus, an early detection of infection with any of the HR HPVs with the help of sensitive molecular tools appears to be necessary component of screening of CaCx, especially in HIV-positive women.

Based on the knowledge of genotype-specific changes in the L1 and E6 region of the viral genome, different investigators have designed in-house PCR/hybridization-based HPV detection and typing methods. There are FDA-approved HPV kits, namely, Hybrid Capture II, Cervista™ HPV HR, Cervista™ HPV-16/-18, Cobas® HPV Test, which are used for the detection of HR HPV in DNA isolated from cervical samples.[17] It has been recently observed in ATHENA study that HPV 16+/18+ women had a greater risk of CIN 2 or worse compared with pooled HR-HPV-positive and HR-HPV-negative women (24.4%, 14.0% and 0.8%, respectively).[31] In the present study, we have tried to devise in-house nested PCR-based protocol for simultaneous detection of mucosal HPVs and typing of the two most common genotypes 16 and 18 in FFPE samples of CaCx and its preinvasive lesions.

The sensitivity of the nested PCR protocol observed in this study is lower in comparison to the observations made by Sotlar *et al*.[16] In their study, Sotlar *et al*. have observed that the E6 nested PCR protocol could detect HPV-specific gene sequence to the level of femtogram (10^{-15} g), whereas in our study, we were able to detect HPV-specific gene sequence to the level of picograms (10^{-12} g). The reason for the lower sensitivity of our protocol might be due to use of whole cell DNA and DNA harvested from SiHa cell line, whereas Sotlar *et al*. have demonstrated greater sensitivity on HPV DNA containing plasmid.

The archival FFPE tissues are rich source of biological materials for genetic analysis. In this study, we have subjected 34 FFPE tissue samples of CaCx and various stages of CIN to NMPCR for simultaneous detection of HPV-16 and 18. Out of 24 FFPE samples of SCC of the uterine cervix, 17 (70.8%) samples yielded 134 bp amplicons of HPV, of which only three samples were found to be positive for HPV-16. The lower positivity of HPV-16 may be due to unavailability of intact 630 bp of E6/7 gene sequence of HPV, possibly because of formalin-induced inhibition of PCR and/or cross-linking of DNA.[32,33] This is further strengthened by our observations of increased detection rate of HPV by GP+/mGP nested PCR which detected 17 of 24 FFPE tissue samples of CaCx, whereas MY/GP+ nested PCR detected only 11 samples. In this study, using the former protocol by employing GP5+/GP6+ as primers for the first round of PCR, we targeted only 142 bp consensus sequence of L1 capsid gene, whereas in the latter MY/GP nested PCR, MY11, and MY09 primers of first round PCR were used to amplify 452 bp sequence, which may not be intact in the sample for amplification. Earlier, Kleter *et al*. designed a PCR-based detection system in which they used a general

primer set, designated SPF1/2 comprised of 10 primers that amplifies a 65-bp segment of the L1 region of the HPV genome, especially suited for FFPE tissue samples that often yields poorly amplifiable DNA. The specific genotype of positive samples is then determined using a reverse hybridization Line Probe Assay.[34]

In this study, we have performed DNA isolation using commercial DNA isolation kit meant for FFPE samples. We have further attempted to develop NMPCR protocol by targeting <200 bp consensus sequence of L1 capsid gene employing GP+/mGP+ primers as well as simultaneous typing of HPV-16/-18 by targeting E6/7 sequence. In this protocol by subjecting the sample for the second round of amplification by employing modified general primers, mGP5+/mGP6+ (designed for the present study), we have attempted to increase the detection rate of HPV from FFPE samples. The first round primers GP5+/GP6+, which were used to target 142 bp L1 capsid gene in NMPCR, were designed earlier by de Roda Husman *et al*. to detect HPV-6, -11, -13, -16, -18, -30, -31, -32, -33, -34, -35, -39, -40, -42, -45, -51, -52, -53, -56, -58, -61, -66 from the list of already described HPVs at that time.[23] They have further observed that despite the presence of primer-template mismatches, successful amplification by consensus sequence PCR can be ensured by the presence of two to three perfectly matching nucleotides at 3' primer ends.

Among these 22 HPVs, 11 namely HPV-16, -18, -31, -33, 35, -39, -45, -51, -52, -56, and -58 are known HR HPVs and a single probable HR (PHR) HPV (HPV-66) from the list of 15 HR and three PHR genotypes described by Muñoz *et al*., including the most common types HPV-16 and -18.[6] In 1995, de Roda Husman *et al*. did not analyze the potential of GP + primers in amplification of HPV-59, -68, -73, and -82 (reason unknown). On *in silico* analysis, we have observed that these genotypes may also get amplified by the GP + primers as these genotypes possess two perfectly matching nucleotides at the 3' primer ends [Figure 3]. However, this observation needs wet laboratory testing for confirmation. Further, the sequencing result of control cell line DNA as well as five selected samples of CaCx not only validates the NMPCR protocol for HPV detection of our study but also highlights the typing capability for the two most common cervical cancer associated HPVs 16 and 18.

CONCLUSIONS

This study indicates the usefulness of our NMPCR protocol for detection of mucosal HPVs and typing of HPV-16 and -18 from FFPE samples of CaCx. This NMPCR protocol may be employed for detection of HPVs and typing the two most common genotypes -16 and -18 in fresh tissue of cervical biopsy or scrape samples for screening of CaCx.

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Conflicts of interest

There are no conflicts of interest.

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